Two Intracellular Pathways Mediate Metabotropic Glutamate Receptor-Induced Ca^{2+} Mobilization in Dopamine Neurons

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Activation of metabotropic glutamate receptors (mGluRs) causes membrane hyperpolarization in midbrain dopamine neurons. This hyperpolarization results from the opening of Ca^{2+}-sensitive K^+ channels, which is mediated by the release of Ca^{2+} from intracellular stores. Neurotransmitter-induced mobilization of Ca^{2+} is generally ascribed to the action of inositol 1,4,5-triphosphate (IP_3) in neurons. Here we show that the mGluR-mediated Ca^{2+} mobilization in dopamine neurons is caused by two intracellular second messengers: IP_3 and cyclic ADP-ribose (cADPR). Focal activation of mGluRs, attained by synaptic release of glutamate or iontophoretic application of aspartate, induced a wave of Ca^{2+} that spread over a distance of ~50 μm through dendrites and the soma. Simultaneous inhibition of both IP_3- and cADPR-dependent pathways with heparin and 8-NH_2-cADPR was required to block the mGluR-induced Ca^{2+} release, indicating a redundancy in the signaling mechanism. Activation of ryanodine receptors was suggested to mediate the cADPR-dependent pathway, because ruthenium red, an antagonist of ryanodine receptors, inhibited the mGluR response only when the cADPR-dependent pathway was isolated by blocking the IP_3-dependent pathway with heparin. Finally, the mGluR-mediated hyperpolarization was shown to induce a transient pause in the spontaneous firing of dopamine neurons. These results demonstrate that an excitatory neurotransmitter glutamate uses multiple intracellular pathways to exert an inhibitory control on the excitability of dopamine neurons.

Key words: **intracellular Ca^{2+} signaling; metabotropic glutamate receptors; inositol 1,4,5-triphosphate; cyclic ADP-ribose; inositol 1,4,5-triphosphate receptors; ryanodine receptors; dopamine neurons; firing pattern**

Introduction

Dopaminergic neurons in the ventral midbrain, i.e., the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc), are a key component of the endogenous reward circuit. They are transiently activated by the detection, perception, and expectation of rewards, suggesting that they are under the control of highly processed inputs from the cerebral cortex and other brain regions (Schultz, 1998). Repetitive stimulation of glutamatergic inputs evokes a slow IPSP via activation of metabotropic glutamate receptors (mGluRs) in dopamine neurons (Fiorillo and Williams, 1998). Release of Ca^{2+} from intracellular stores mediates this mGluR-induced hyperpolarization, because the rise in $[Ca^{2+}]_i$ will activate small-conductance Ca^{2+}-sensitive K^+ channels (SK channels) on the plasma membrane. The resulting prolonged hyperpolarization (~1 sec) is expected to have a significant impact on the firing pattern of dopamine neurons.

It is generally assumed that inositol 1,4,5-triphosphate (IP_3) mediates the mobilization of Ca^{2+} induced by activation of G-protein-linked neurotransmitter receptors in neurons (Berridge, 1998). Recent studies have reported that synaptically released glutamate acting on mGluRs evokes IP_3-mediated mobilization of Ca^{2+} in cerebellar Purkinje neurons and hippocampal pyramidal neurons (Finch and Augustine, 1998; Takechi et al., 1998; Nakamura et al., 1999, 2000). In dopamine neurons, direct application of IP_3 into the cytosol has been shown to elicit release of Ca^{2+} from intracellular stores (Morikawa et al., 2000), consistent with the involvement of IP_3 in the mGluR-induced Ca^{2+} signal.

Cyclic ADP-ribose (cADPR) is another Ca^{2+}-releasing messenger in mammalian systems (Petersen and Cancela, 1999). The Ca^{2+}-mobilizing activity of cADPR was described originally in sea urchin eggs (Lee et al., 1989). Recent studies have shown that it is also involved in the cell surface receptor-mediated Ca^{2+} signals in pancreatic acinar cells and T-lymphocytes (Cancela et al., 1999; Guse et al., 1999). In the nervous system, cADPR has been found to potentiate Ca^{2+}-induced Ca^{2+} release via ryanodine receptors and to enhance neurotransmitter release from presynaptic terminals (Hua et al., 1994; Empson and Galione, 1997; Mothet et al., 1998; Brailoiu and Miyamoto, 2000). However, the role of cADPR in mediating neurotransmitter-elicited Ca^{2+} release has never been demonstrated in neurons.

In this study, the intracellular second messenger cascade mediating the release of Ca^{2+} after activation of mGluRs in dopamine neurons was investigated using confocal imaging of $[Ca^{2+}]_i$ combined with whole-cell recording of the membrane conductance. The results obtained show that both IP_3 and cADPR mediate mGluR-induced Ca^{2+} mobilization.

Received July 23, 2002; revised Sept. 16, 2002; accepted Oct. 22, 2002.

This work was supported by National Institutes of Health Grant DA04523. We thank Olivier Manzoni for comments on this manuscript.

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 0270-6474/02/220149-08$15.00/0

Materials and Methods

Slices and solutions. Horizontal slices (180–220 μm) of the ventral midbrain were prepared from adult Wistar rats (160–220 gm). Slices were cut using a Vibratome (Leica) in an ice-cold physiological saline containing (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose, 21.4 $NaHCO_3$, saturated with 95% O_2 and 5% CO_2, pH 7.4, 300 mOsm/kg, and then stored in the same solution warmed to 35°C for at least 30 min. For recordings, a single slice was placed in a recording chamber and superfused with the warmed (35°C) physiological saline at 1–2 ml/min. Unless noted otherwise, pipette solutions used for whole-cell and cell-attached recordings contained (in mM): 115 K-methylsulfate, 20 KCl, 1.5 $MgCl_2$, 10 HEPES, 0.1 EGTA, 2 Mg-ATP, 0.2 Na_2-GTP, and 10 Na_2 phosphocreatine, pH 7.3, 275 mOsm/kg.

Whole-cell recording. All recordings were performed in dopamine neurons, which were identified by their large cell bodies (~20 μm), the characteristic pacemaker-like firing (1–5 Hz) observed in the cell-attached mode, and the presence of a large (>200 pA) I_H current. Cells were visualized using a 40 or 60× water-immersion objective on an upright microscope (Zeiss) with infrared illumination. Whole-cell pipettes had resistances of 1.5–3 MΩ. Voltage-clamp recordings were made, and the holding potential was routinely set at −55 mV. An Axopatch 1D amplifier (Axon Instruments, Foster City, CA) was used to record the data, which were filtered at 1 kHz, digitized at 5 kHz, and collected on a personal computer using AxoGraph 4 (Axon Instruments).

Cell-attached recording. The firing was monitored with the cell-attached mode, because the spontaneous firing of dopamine neurons was significantly distorted with the whole-cell recording. Usage of low-resistance pipettes (1.5–1.8 MΩ) together with the formation of a large Ω-shape membrane invagination allowed enough access to the cell interior to monitor the membrane potential with a current-clamp recording.

Ca^{2+} imaging. Fluorescence imaging was made with the whole-cell recording configuration using a pipette solution containing Oregon Green 488 BAPTA-2 (50 μM). Images were taken at 15 Hz for 3–5 sec using a confocal imaging system (Solamere Technology). Ca^{2+} signals from selected regions of interest (ROIs) were expressed as fractional change in fluorescence, $\%\Delta F/F = 100 \times (F - F_{baseline})/(F_{baseline} - F_{background})$. The inclusion of heparin and 8-NH_2-cADPR in the pipette did not affect the calcium signal induced by depolarization to 0 mV (200 msec) to cause Ca^{2+} influx. In this control experiment, the increase in fluorescence was determined at 5 and 15 min after the onset of whole-cell recording, and the 15 min/5 min ratio was 1.3 ± 0.2 ($n = 3$).

Evoked mGluR-mediated responses. The mGluR-mediated release of Ca^{2+} is extremely vulnerable to rapid desensitization. Thus, agonists must be applied very rapidly to observe the response. This was achieved either by electrical stimulation of presynaptic fibers or by iontophoresis of aspartate. Synaptic responses were evoked with a bipolar tungsten stimulating electrode (tip separation 50–100 μm), which was placed at 30–100 μm to the recorded cell. A train of 5–10 stimuli were applied at 66 Hz to evoke the mGluR-mediated synaptic response. Iontophoresis was performed with an Axoclamp 2A amplifier (Axon Instruments) (up to 200 nA ejection current, 5–20 nA backing current) using small-tipped pipettes (40–100 MΩ) containing 1 M aspartate. Iontophoretic pipettes were placed within 5 μm of the soma or dendrites. The direction of the pipette was made at, or close to, right angles to the longitudinal direction of the cell to ensure that aspartate was applied focally to the cell. Experiments were done in the presence of 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (5 μM), and the slices were pretreated with (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (50–100 μM) to block AMPA- and NMDA-mediated responses. In experiments in which synaptic responses were elicited, picrotoxin (100 μM), CGP 35348 (100 μM), strychnine (1 μM), and eticlopride (100 nM) were further added to block $GABA_A$, $GABA_B$, glycine, and dopamine D_2 receptors.

Flash photolysis of caged IP_3. Whole-cell recordings were performed with intracellular solutions containing caged IP_3 (200 μM). A xenon arc lamp (Cairn Research) was used to produce UV pulses (~1 msec). The capacitance and the voltage of the capacitor supplying current to the flash lamp were set at 4000 μF and 300 V, respectively. This evoked a near-maximal outward current in each cell.

Drugs. Drugs were applied either by extracellular perfusion or intracellular dialysis through the whole-cell pipette. A-methyl-4-carboxyphenylglycine [(*S*)-MCPG], 7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt), dihydroxyphenylglycine [(*S*)-3,5-DHPG], 2-amino-5-phosphonopentanoic acid (D-AP-5), and NBQX were obtained from Tocris Cookson. Thapsigargin and ryanodine were obtained from Calbiochem (LaJolla, CA). Ruthenium red was obtained from Alomone Labs. Caged IP_3, 8-NH_2-cADPR, and Oregon Green 488 BAPTA-2 were obtained from Molecular Probes (Eugene, OR). CGP 35348 was a gift from Novartis. All other chemicals were obtained from Sigma (St. Louis, MO)/RBI (Natick, MA).

Data analysis. Data are expressed as mean ± SEM. Statistical significance was determined with Student's *t* test or one-way ANOVA followed by the *post hoc* Dunnett's test. The difference was considered significant at $p < 0.05$.

Results

mGluR-induced wave of Ca^{2+}

Whole-cell recordings of synaptic responses were made from dopamine neurons in rat midbrain slices. To isolate the mGluR-mediated responses, AMPA, NMDA, $GABA_A$, $GABA_B$, glycine, and dopamine D_2 receptors were blocked pharmacologically. $[Ca^{2+}]_i$ was monitored by the change in fluorescence of Oregon Green 488 BAPTA-2 (50 μM) loaded into the cell through the whole-cell pipette. A train of 5–10 stimuli (66 Hz) with an extracellular bipolar stimulating electrode (tip separation 50–100 μm) evoked an increase in $[Ca^{2+}]_i$ and an outward current ($n = 11$) (Fig. 1*A*). These synaptic responses were inhibited by CPCCOEt (50–75 μM; $n = 4$), an mGluR antagonist. The rise in $[Ca^{2+}]_i$ invariably originated in dendrites 10–50 μm away from the soma and propagated bi-directionally as a wave. The Ca^{2+} wave spread over a distance of 20–50 μm from the origin, reaching the soma in most cases. The speed of wave propagation over the initial 15–20 μm was 111 ± 29 μm/sec ($n = 11$) (see also Fig. 4*C*). Frequently, multiple waves were observed either in a same dendrite or in different dendrites and appeared to collide with one another (Fig. 1*A*), complicating the analysis of wave propagation.

Next, focal application of aspartate was made with iontophoresis (50–200 msec). In these experiments, AMPA- and NMDA-mediated responses were blocked with antagonists. Aspartate iontophoresis also evoked a wave-like increase in $[Ca^{2+}]_i$, which originated at the site of application (supplemental material), that was accompanied with an outward current ($n = 15$) (Fig. 1*B*). Both responses were inhibited by the mGluR antagonists MCPG (1 mM; $n = 2$) and CPCCOEt (50–75 μM; $n = 6$). Furthermore, both were desensitized by perfusion with a low concentration of DHPG (1 μM; $n = 10$), an mGluR agonist. Thus, aspartate iontophoresis induced a focal activation of mGluRs to elicit a Ca^{2+} wave and an outward current.

Aspartate could be applied anywhere on the cell (both the soma and dendrites) to elicit a Ca^{2+} wave. The aspartate iontophoresis-induced wave traveled over a longer distance (40–80 μm) than the synaptic wave. However, the speed of propagation over the initial 15–20 μm from the origin (121 ± 16 μm/sec; $n = 15$) was similar to the synaptic wave ($p > 0.05$) (see Fig. 4*C*). The magnitude and the rate of the rise in $[Ca^{2+}]_i$, as well as the speed of propagation, decreased significantly as the wave spread away from the origin. In 12 cells in which the wave traveled >45 μm, the propagation speed measured between the origin and 15–20 μm was 123 ± 24 μm/sec. The propagation speed declined to 35 ± 4 μm/sec measured beyond 30 μm from the origin ($p < 0.005$). Thus, it is suggested that a diffusive process contributes to the overall wave propagation.

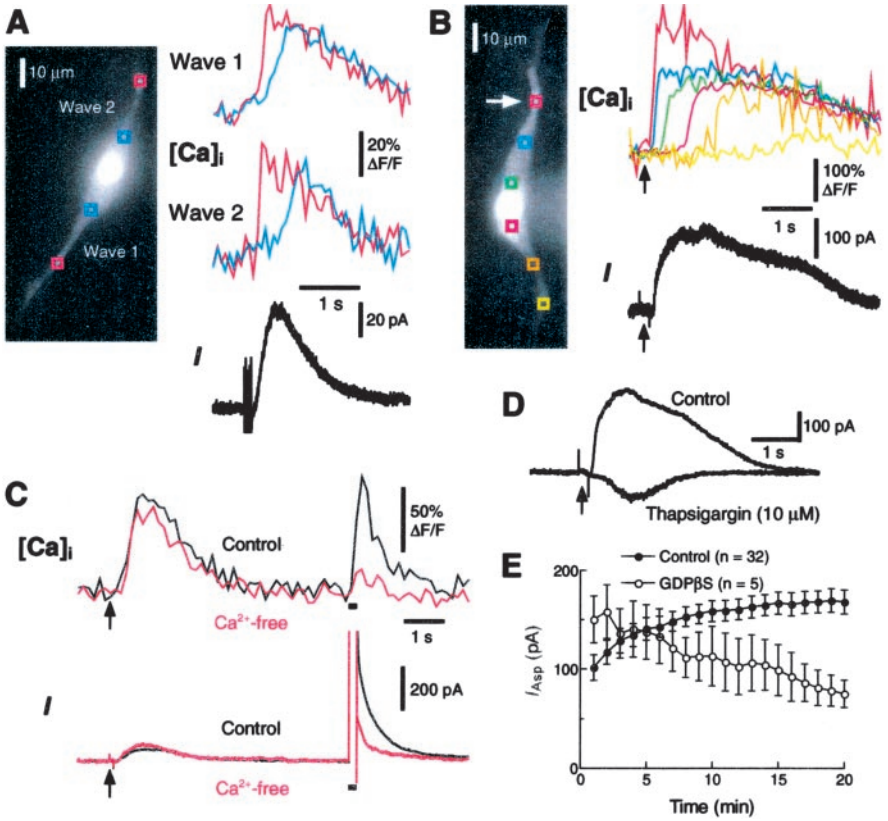

Figure 1. Focal activation of mGluRs induces a wave of Ca^{2+}. *A*, Extracellular synaptic stimulation induced waves of Ca^{2+} originating in dendrites. A confocal fluorescence image of a dopamine neuron loaded with Oregon Green 488 BAPTA-2 (50 μM) is shown on the *left*. Fluorescence changes were measured at the ROIs placed at the origins of waves and 20 μm away from the origins toward the soma. Two Ca^{2+} waves (*Wave 1* and *Wave 2*) were elicited in opposite dendrites. The IPSC evoked concomitantly is also shown. *B*, Focal application of aspartate elicited a wave of Ca^{2+} originating at the application site. A confocal fluorescence image of a dopamine neuron loaded with Oregon Green 488 BAPTA-2 (50 μM) is shown on the *left*. Aspartate was iontophoresed at the site indicated by the *arrow*. Fluorescence changes were measured at the ROIs placed every 15 μm starting at the application site. The outward current evoked by aspartate in the same cell is also shown. Aspartate was iontophoresed at the time indicated by *arrows*. *C*, The rise in $[Ca^{2+}]_i$ and the outward current elicited by aspartate were insensitive to removal of Ca^{2+} from the extracellular solution for 5 min. The same treatment nearly abolished the rise in $[Ca^{2+}]_i$ and the outward current after a depolarization (200 msec) to 0 mV (*horizontal bar*). *D*, The aspartate-evoked outward current was abolished by thapsigargin (10 μM). *E*, The aspartate-evoked outward current is plotted against time after going into the whole-cell mode. Recordings were made with either a control internal solution (●) or a solution with GDPβS (3 mM) (○).

The rise in $[Ca^{2+}]_i$ and the outward current evoked by aspartate were insensitive to removal of Ca^{2+} from the extracellular solution ($n = 6$) (Fig. 1*C*). Furthermore, the aspartate-evoked outward current was completely abolished by thapsigargin (10 μM; $n = 3$) (Fig. 1*D*), which depletes intracellular Ca^{2+} stores by blocking the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1990). Inhibiting the outward current with thapsigargin revealed an aspartate-evoked inward current. It has been shown that this inward current is also mediated by activation of mGluRs but is independent of Ca^{2+} mobilization (Guatteo et al., 1999). These results indicate that the rise in $[Ca^{2+}]_i$ originates from intracellular stores.

The involvement of G-proteins was examined next by applying GDPβS (3 mM), a general inhibitor of G-protein function, intracellularly through the whole-cell pipette. The outward current was monitored as readout of $[Ca^{2+}]_i$. In control, the amplitude of the aspartate-induced outward current increased over the first 10 min of recording and stabilized, averaging 169 ± 12 pA at 20 min ($n = 32$) (Fig. 1*E*). In contrast, the current gradually declined when GDPβS (3 mM) was included in the pipette solution and averaged 75 ± 14 pA ($n = 5$; $p <$ 0.05 vs control) after 20 min. Furthermore, intracellular application of GTPγS (200 μM), which tonically activates G-proteins, completely occluded, or desensitized, the outward current in 1–3 min ($n = 5$) (data not shown). These results demonstrate that G-proteins are involved in the mGluR-mediated release of Ca^{2+}.

IP_3 is not the sole intracellular messenger responsible for the mGluR-mediated release of Ca^{2+}

Application of IP_3 directly into the cytosol has been shown to induce release of Ca^{2+} from intracellular stores and subsequent activation of SK channels in dopamine neurons (Morikawa et al., 2000). Furthermore, it has been shown that the IP_3-evoked outward current is desensitized by superfusion of a low concentration of an mGluR agonist (Paladini et al., 2001). Thus, IP_3 has been suggested to be the intracellular messenger responsible for the mGluR-mediated release of Ca^{2+}. To directly test this idea, heparin, a competitive antagonist of IP_3 receptors (Ghosh et al., 1988), was used. The effect of heparin on the outward current evoked by flash photolysis of caged IP_3 (200 μM) loaded into the cytosol was examined first. Photolytic release of IP_3 produced a transient outward current, as reported previously (Morikawa et al., 2000). The peak amplitude of the IP_3-evoked current reached a plateau in 10–20 min after the onset of recording (228 ± 34 pA; $n = 6$) (Fig. 2*A*). Inclusion of heparin (1 mg/ml) in the internal solution completely blocked the IP_3-evoked current within 10 min after breaking in (3 ± 2 pA; $n = 6$; $p < 0.0001$ vs control), indicating that heparin is an effective antagonist of IP_3 receptors in dopamine neurons. In contrast, intracellular dialysis of heparin (1 mg/ml) failed to significantly affect the aspartate-induced current (136 ± 17 pA; $n =$ 10; $p > 0.05$ vs control) (Fig. 2*B*). These results strongly suggest that an intracellular messenger besides IP_3 causes Ca^{2+} mobilization after mGluR activation.

Continuous activation of mGluRs desensitizes both mGluR- and IP_3-induced responses (Paladini et al., 2001). To gain further insight into the intracellular mechanism involved, the interaction of the mGluR-mediated and the IP_3-evoked responses was investigated by sequentially applying aspartate with iontophoresis and IP_3 with flash photolysis of caged IP_3 (200 μM). In the cell shown in Figure 2*C*, the outward current produced by photolytic release of IP_3 was completely desensitized when aspartate was applied 5 sec before; however, aspartate still evoked a considerable outward current at 5 sec after aspartate iontophoresis in the same cell. The same observation was made in two other cells. Furthermore, photolytic release of IP_3 2–5 sec before aspartate iontophoresis failed to completely desensitize the aspartate-elicited current in four

cells tested (Fig. 2*D*). These results are consistent with the idea that the mGluR-mediated response involves an IP_3-independent mechanism.

Both IP_3 and cADPR pathways mediate the mGluR-induced release of Ca^{2+}

To search for an alternative intracellular mechanism, 8-NH_2-cADPR, a specific cADPR antagonist (Walseth and Lee, 1993), was used. The effects of heparin and 8-NH_2-cADPR were tested on mGluR IPSCs. Recordings were first made with a pipette containing a control internal solution to obtain a control IPSC amplitude (Fig. 3*A*). In this way, the stimulus intensity was optimized in each cell. After the first pipette was removed from the cell, a second pipette containing a control internal solution or a solution with heparin (1 mg/ml) alone, 8-NH_2-cADPR (50 μM) alone, or both heparin and 8-NH_2-cADPR was patched onto the same cell, and IPSCs were evoked with the same stimulus intensity. The amplitude of the IPSC in the first recording (~10 min after the onset; $IPSC_1$) was used to normalize the IPSC amplitude obtained with the second pipette ($IPSC_2$). The normalized IPSC amplitude ($IPSC_2/IPSC_1$) measured 20 min after the onset of the second recording was similar when the second pipette contained the control solution, heparin alone, or 8-NH_2-cADPR alone (Fig. 3*B*). The IPSP was significantly smaller only in the presence of both heparin and 8-NH_2-cADPR (0.11 ± 0.04, $n = 4$, vs 1.47 ± 0.18 with control solution, $n = 3$; $p < 0.001$) (Fig. 3*A*,*B*).

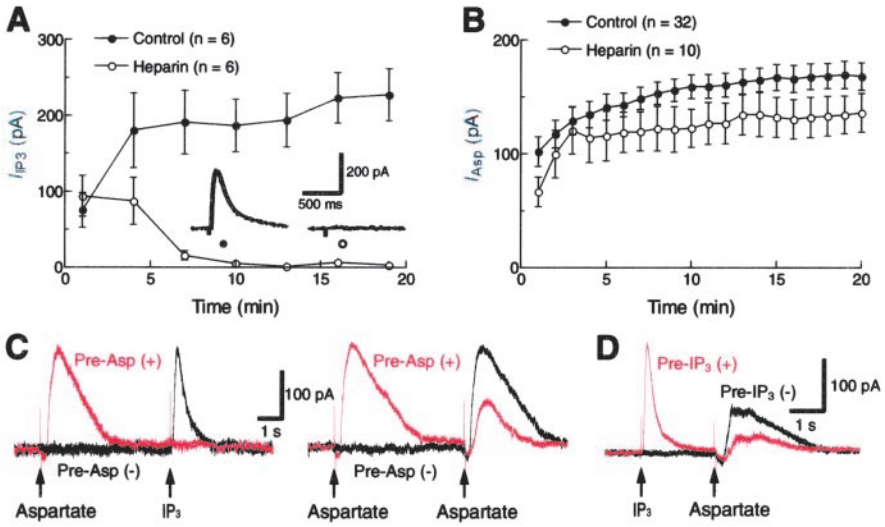

Figure 2. IP_3 receptors do not entirely mediate the mGluR-induced outward current. *A*, *B*, The outward current evoked by flash photolysis of caged IP_3 (200 μM) (*A*) or iontophoretic application of aspartate (*B*) is plotted against time after going into the whole-cell mode. Recordings were made with either a control internal solution (●) or a solution with heparin (1 mg/ml) (○). Representative traces of IP_3-evoked currents are shown as an *inset* in *A*. *C*, Previous iontophoresis of aspartate completely desensitized the outward current evoked by flash photolysis of caged IP_3 (200 μM) (*left traces*). The interval between aspartate iontophoresis and photolytic release of IP_3 was 5 sec. In the same cell, previous iontophoresis of aspartate failed to completely desensitize the outward current elicited by a second application of aspartate 5 sec later (*right traces*). The *black traces* [*Pre-Asp* (−)] are from recordings in which the first application of aspartate was omitted, whereas the *red traces* [*Pre-Asp* (+)] are from recordings in which the aspartate iontophoresis preceded the photolytic release of IP_3 (*left trace*) or the second aspartate iontophoresis (*right trace*). *D*, Previous photolytic release of IP_3 failed to completely desensitize the outward current induced by aspartate 2 sec later. Aspartate iontophoresis and flash photolysis of caged IP_3 were made at the times indicated by *arrows*. Photolytic release of IP_3 was omitted in the *black trace* [*Pre-IP*$_3$ (−)], whereas it was made 2 sec before aspartate iontophoresis in the *red trace* [*Pre-IP*$_3$ (+)].

The effect of 8-NH_2-cADPR was also examined on the aspartate-evoked responses. Intracellular dialysis of 8-NH_2-cADPR (50 μM) alone had no significant effect on the aspartate-evoked current (136 ± 19 pA, $n = 22$, $p > 0.05$ vs control) (Fig. 4*A*). However, inclusion of heparin (1 mg/ml) together with 8-NH_2-cADPR (50 μM) in the pipette abolished the aspartate-evoked outward current and turned it into inward in 8 of 11 cells tested. On average, the current amplitude in the presence of both heparin and 8-NH_2-cADPR was -14 ± 12 pA (inward; $n = 11$, $p < 0.001$ vs control) at 20 min. The effects of heparin and 8-NH_2-cADPR were also tested on the rise in $[Ca^{2+}]_i$ produced by aspartate. Here, the rise in $[Ca^{2+}]_i$ at the soma was first measured with a control solution containing Oregon Green 488 BAPTA-2 (50 μM) (Fig. 4*B*). Then, the pipette was withdrawn, and a second pipette containing both heparin (1 mg/ml) and 8-NH_2-cADPR (50 μM) together with Oregon Green 488 BAPTA-2 was used to make a second whole-cell recording from the same cell. In the second recording, the rise in $[Ca^{2+}]_i$ was reduced to <10% of control after 15–20 min in all three cells tested. When the second pipette contained only the calcium indicator, the rise in $[Ca^{2+}]_i$ was not significantly different from the first recording (second/first $= 1.0 \pm 0.2$; $n = 4$). Aspartate elicited a Ca^{2+} wave in the presence of either heparin or 8-NH_2-cADPR alone that had a propagation velocity that was not significantly different from the wave with a control solution (Fig. 4*C*).

Taken together, these results demonstrate that the mGluR-mediated Ca^{2+} mobilization involves two pathways mediated by IP_3 and cADPR in a redundant manner.

Ryanodine receptors mediate the cADPR pathway

cADPR is known to act on ryanodine receptors (Galione et al., 1991; Meszaros et al., 1993). To examine the involvement of ryanodine receptors in the cADPR-dependent pathway, ruthenium red, an antagonist of ryanodine receptors (Smith et al., 1988), was tested. Intracellular dialysis of ruthenium red (50 μM) alone had no significant effect on the aspartate-evoked current (123 ± 11 pA, $n = 6$, $p > 0.05$ vs control) (Fig. 5*A*). However, when the IP_3-dependent pathway was blocked with heparin (1 mg/ml), intracellular ruthenium red significantly suppressed the aspartate-evoked current (59 ± 12 pA, $n = 8$, $p < 0.001$ vs control). The small outward current remaining in the presence of both heparin and ruthenium red may be caused by an incomplete blockade of ryanodine receptors by ruthenium red. These results show that the cADPR pathway is dependent on the activation of ryanodine receptors.

To further confirm the involvement of ryanodine receptors, the effect of ryanodine was examined next. Ryanodine locks the ryanodine receptor channel in a subconductance open state (Rousseau et al., 1987). Thus, ryanodine blocks the ryanodine receptor-dependent Ca^{2+} signal in two ways by (1) preventing the full activation of ryanodine receptors and (2) gradually depleting the stores expressing ryanodine receptors, whereas the IP_3-induced Ca^{2+} release will be inhibited by ryanodine only via the second mechanism, i.e., store depletion, if the IP_3-sensitive stores coexpress ryanodine receptors (Khodakhah and Armstrong, 1997; Morikawa et al., 2000). Therefore, the ryanodine receptor-mediated Ca^{2+} signal is expected to be more sensitive to

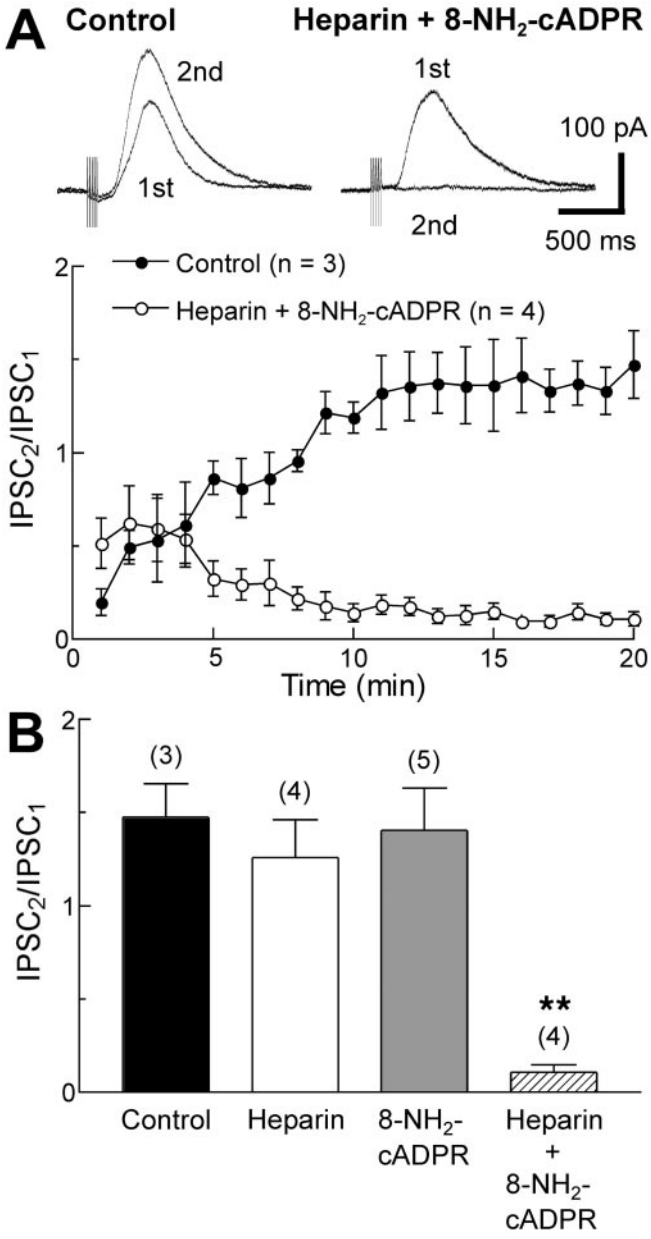

Figure 3. Simultaneous blockade of IP_3- and cADPR-induced signaling inhibits mGluR IPSCs. *A*, Intracellular dialysis of both heparin (1 mg/ml) and 8-NH_2-cADPR (50 μM) nearly abolished mGluR IPSCs. The cell was first recorded with a control internal solution to obtain a control IPSC amplitude ($IPSC_1$) at ~10 min after the onset of recording. The same cell was subsequently patched with a pipette containing a control solution or a solution with both heparin and 8-NH_2-cADPR. IPSCs were recorded by applying the same train of stimuli used for the first patch ($IPSC_2$) and plotted after they were normalized by the $IPSC_1$. Representative *traces* are shown above. *B*, The normalized IPSC amplitudes at 20 min of recording are shown for the second patch with a control solution or a solution containing heparin (1 mg/ml) alone, 8-NH_2-cADPR (50 μM) alone, or both heparin and 8-NH_2-cADPR.

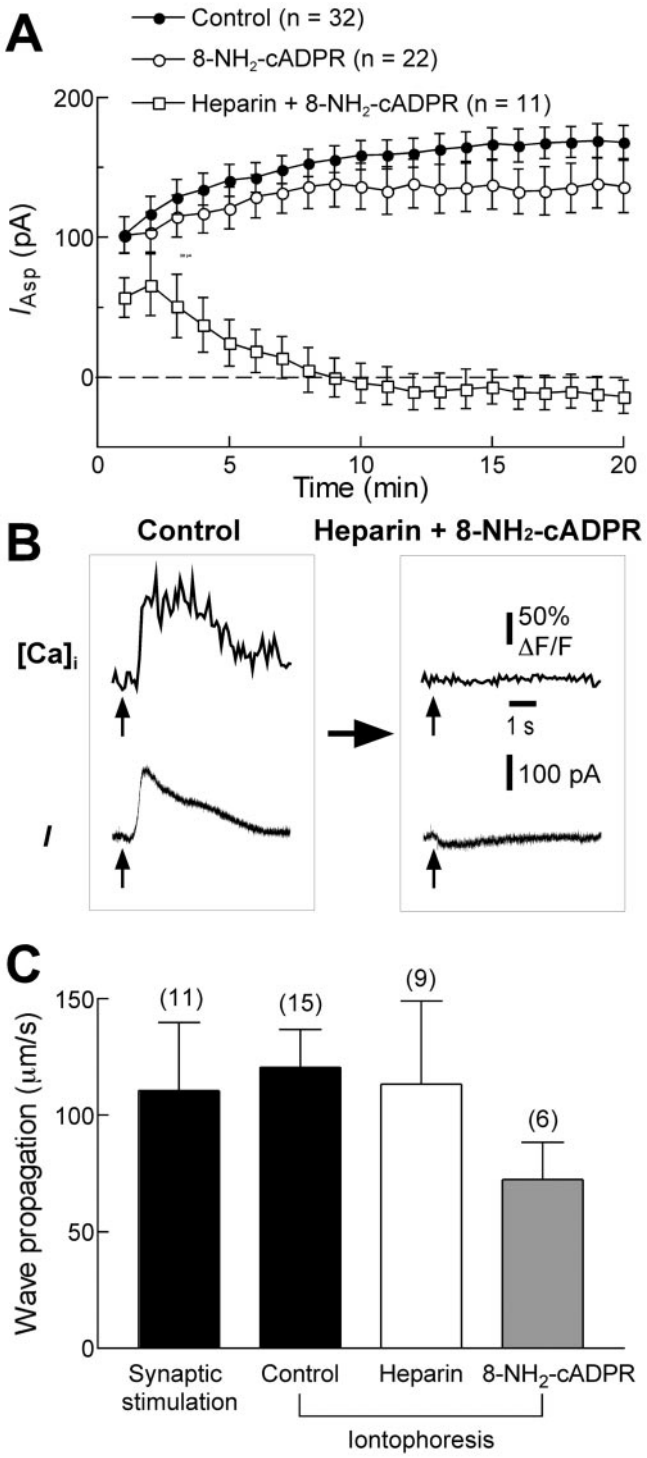

Figure 4. Simultaneous blockade of IP_3- and cADPR-induced signaling inhibits mGluR-mediated responses. *A*, The aspartate-induced outward current is plotted versus time after going into the whole-cell mode. Recordings were made with a pipette containing a control internal solution (●), 8-NH_2-cADPR (50 μM) (○), or both heparin (1 mg/ml) and 8-NH_2-cADPR (50 μM) (□). *B*, *Traces* of the rise in $[Ca^{2+}]_i$ and the outward current evoked by aspartate iontophoresis. Recordings on the *left* were done with control internal solution containing Oregon Green 488 BAPTA-2 (50 μM). The *traces* on the *right* were from a second recording in the same cell using a pipette containing both heparin (1 mg/ml) and 8-NH_2-cADPR (50 μM) in addition to Oregon Green 488 BAPTA-2. *C*, A *bar graph* showing the speed of propagation at the initial 15–20 μm from the origin for the Ca^{2+} waves induced by synaptic stimulation and aspartate iontophoresis. For aspartate iontophoresis, the speed is shown for the recordings with a control internal solution and a solution with either heparin (1 mg/ml) alone or 8-NH_2-cADPR (50 μM) alone.

ryanodine, because ryanodine can more directly block it even before store depletion. Bath perfusion of ryanodine (10 μM) for 20 min reduced the aspartate-evoked current by 44 ± 6% (n = 12) (Fig. 5*B*,*C*). When the IP_3-dependent pathway was blocked with heparin (1 mg/ml), the rate of ryanodine block was accelerated, and ryanodine nearly abolished the aspartate-induced current after 20 min (99 ± 12% inhibition, $n = 5$, $p < 0.001$ vs control). On the other hand, when the cADPR-dependent pathway was blocked with 8-NH_2-cADPR (50 μM), ryanodine pro-

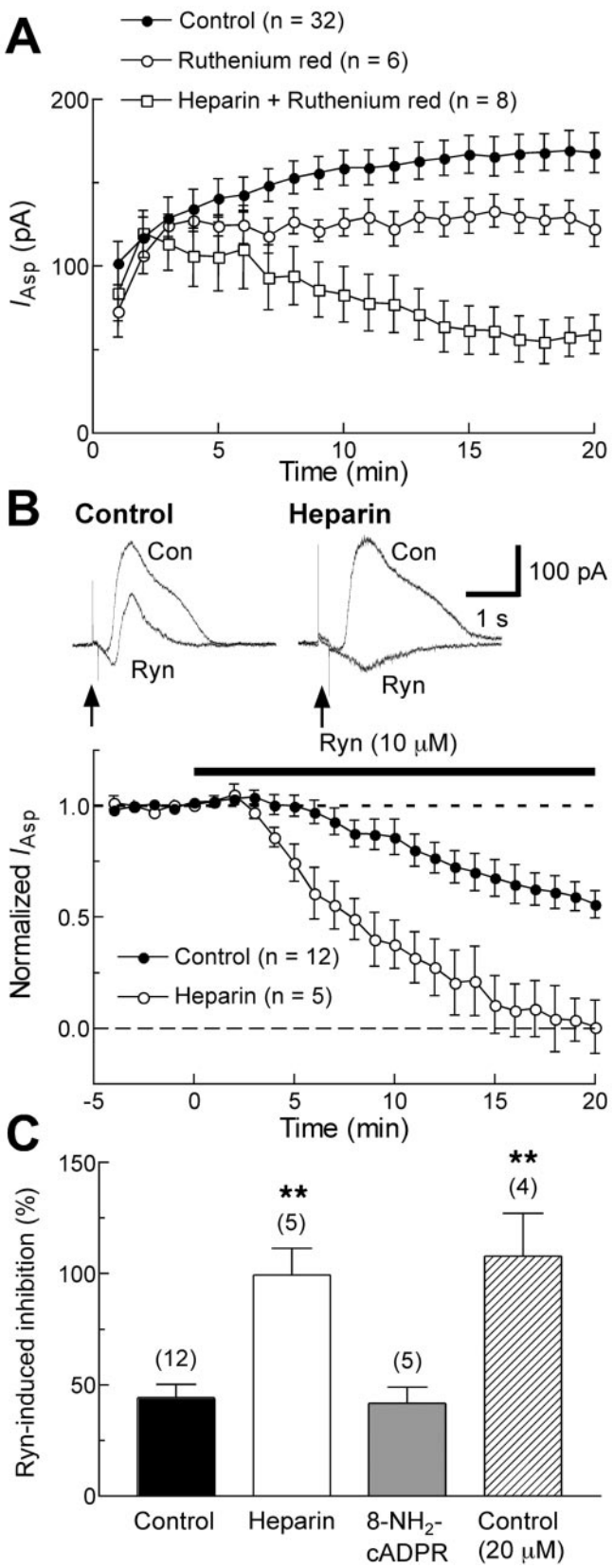

Figure 5. Ryanodine receptors mediate the cADPR pathway. *A*, The aspartate-evoked outward current is plotted versus time after the onset of recording. Recordings were made with a pipette containing a control internal solution (●), ruthenium red (50 μM) (○), or both heparin (1 mg/ml) and ruthenium red (50 μM) (□). *B*, A summary time graph showing the effect of ryanodine (10 μM) on the aspartate-evoked outward current. Recordings were done with a control internal solution (●) or a solution with heparin (1 mg/ml) (○). Ryanodine was perfused for 20 min at the time indicated by the *bar*. The data are shown after the amplitude of the aspartate-induced current had reached a steady state (15–20 min after the onset of whole-cell recording). Current amplitude was normalized to the mean amplitude over a 5 min period before ryanodine application. Representative *traces* before and after ryanodine application are shown above. *C*, A summary *bar graph* showing the effect of ryanodine (10 μM) on the aspartate-induced current recorded with a control internal solution and a solution with either heparin (1 mg/ml) or 8-NH_2-cADPR (50 μM). The *hatched bar* shows the effect of ryanodine (20 μM) on the current with a control internal solution. $^{**}p < 0.01$.

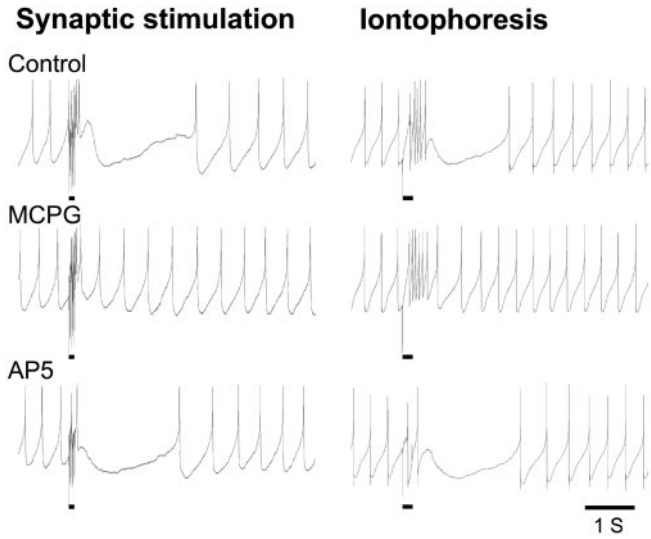

Figure 6. mGluR-mediated hyperpolarization induces a pause after burst firing. The firing pattern of a dopamine neuron is shown in the control condition, in the presence of MCPG (1 mM), and in the presence of AP-5 (50 μM). Repetitive synaptic stimulation (8 stimuli at 66 Hz; *left traces*) or aspartate iontophoresis (200 msec; *right traces*) were made at the times indicated by *bars*.

duced an inhibition of the aspartate-evoked current comparable with that of the control current (42 ± 7% inhibition, $n = 5$, $p > 0.05$ vs control). Hence, the cADPR-dependent pathway was significantly more sensitive to the ryanodine blockade than the IP_3-dependent pathway ($p < 0.001$), which is consistent with the involvement of ryanodine receptors in the cADPR-dependent pathway. Increasing the concentration of ryanodine to 20 μM completely blocked the control aspartate-induced current in 20 min (108 ± 19% inhibition; $n = 4$) (Fig. 5*C*). The accelerated blockade achieved by the higher concentration of ryanodine (20 μM) was most likely caused by the accelerated depletion of IP_3-sensitive stores coexpressing ryanodine receptors. In this series of experiments, the calculated inhibition of the outward current is likely to be a slight overestimate of the true value, because the outward current is riding on top of an inward current.

mGluR-mediated hyperpolarization induces a pause of firing

The effect of extracellular synaptic stimulation or aspartate iontophoresis on the firing was examined next. These experiments were done in the absence of AMPA and NMDA antagonists. In the case of synaptic stimulation, $GABA_A$, $GABA_B$, glycine, and dopamine D_2 receptors were blocked with respective antagonists. The firing was monitored with a cell-attached configuration. Dopamine neurons displayed a spontaneous pacemaker-type firing with a firing frequency of 1–5 Hz, as reported previously (Fig. 6) (Grace and Onn, 1989). Repetitive extracellular stimulation (8–10 stimuli at 66 Hz) induced a pause of firing lasting 1–2 sec in

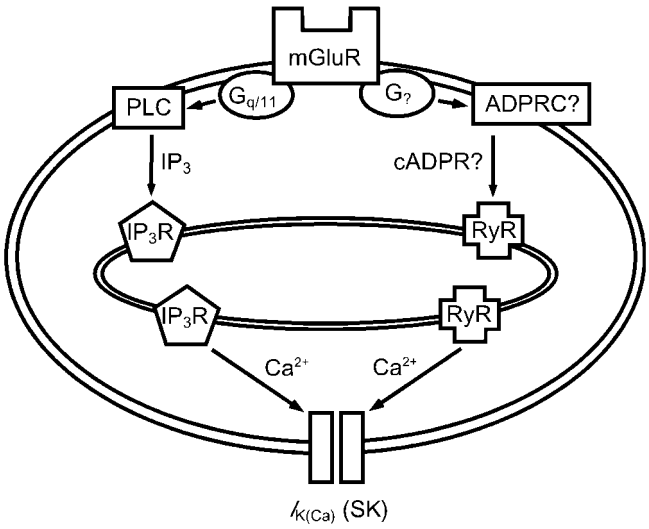

Figure 7. Proposed intracellular signaling cascade mediating the mGluR-induced Ca^{2+} mobilization. Activation of mGluRs leads to dual activation of phospholipase C (*PLC*) and ADP-ribosyl cyclase (*ADPRC*). PLC catalyzes the production of IP_3, whereas ADPRC catalyzes the production of cADPR. IP_3 and cADPR induce release of Ca^{2+} via IP_3 receptors (*IP_3R*) and ryanodine receptors (*RyR*), respectively. IP_3Rs and RyRs are coexpressed on a common Ca^{2+} pool.

all five cells tested. A burst of firing lasting ~200 msec with a firing frequency of ~20 Hz preceded the pause in two of five cells. Iontophoretic application of aspartate (50–200 msec) invariably induced a burst firing (10–20 Hz for 200–500 msec) followed by a pause lasting 0.5–5 sec ($n = 10$). Perfusion of MCPG (1 mM), an mGluR antagonist, dramatically attenuated the pause ($n = 2$ for synaptic stimulation, $n = 3$ for iontophoresis). Furthermore, perfusion with a low concentration of DHPG (1 μM), an mGluR agonist, also reversibly inhibited the pause ($n = 3$ for synaptic stimulation, $n = 5$ for iontophoresis) by desensitizing the mGluR-mediated hyperpolarization. On the other hand, bath application of AP-5 (50 μM), an NMDA antagonist, reversibly inhibited the burst without affecting the pause ($n = 2$ for synaptic stimulation, $n = 5$ for iontophoresis). Therefore, synaptic release of glutamate, as well as aspartate iontophoresis, evoked an NMDA receptor-dependent burst followed by a pause caused by the mGluR-mediated hyperpolarization.

Discussion

This study demonstrates that mGluRs can release Ca^{2+} in dopamine neurons through the activation of both IP_3 and ryanodine receptors. The activation of either receptor alone is sufficient to cause the release of Ca^{2+}. IP_3 and cADPR most likely mediate the activation of IP_3 and ryanodine receptors, respectively (Fig. 7). This is in accord with previous reports showing that the fertilization-induced Ca^{2+} wave in sea urchin eggs is mediated by a redundant mechanism involving both IP_3 and cADPR (Galione et al., 1993; Lee et al., 1993). Finally, mGluR-induced hyperpolarization resulting from the rise in $[Ca^{2+}]_i$ is shown to mediate the pause of firing that acts to curtail the burst firing caused by NMDA receptor activation. This observation would provide a cellular mechanism responsible for the burst–pause type of firing observed *in vivo*.

A wave of Ca^{2+}

In the present study, both synaptic release of glutamate and focal application of aspartate evoked a wave of Ca^{2+}. Repetitive stimulation of glutamatergic fibers was necessary to elicit a Ca^{2+} wave, suggesting that extrasynaptic spillover of glutamate may mediate the activation of postsynaptic mGluRs (Brasnjo and Otis, 2001). In line with this, group I mGluRs have been located at extrasynaptic sites in the SNc (Hubert et al., 2001). The aspartate-induced wave initiated at the application site regardless of the placement of the iontophoretic pipette, indicating that the machinery necessary for the mGluR-mediated Ca^{2+} mobilization is present throughout the cell. On the other hand, the origin of the synaptic wave was invariably located in dendrites. Glutamatergic synapses are widely distributed in dopamine neurons, on both the soma and dendrites (Smith et al., 1996). Multiple glutamatergic fibers synapsing on both the soma and dendrites were most likely stimulated in the present study, because the bipolar stimulating electrode used had a wide tip separation (50–100 μm). Therefore, it is possible that only the synapses on dendrites are associated with sufficient levels of perisynaptic mGluRs to induce the release of Ca^{2+}. Indeed, mGluRs are expressed more densely in dendrites than in the soma in dopamine neurons (Kosinski et al., 1998).

The mGluR-induced Ca^{2+} signal has different spatiotemporal profiles in different neurons (Finch and Augustine, 1998; Takechi et al., 1998; Nakamura et al., 1999, 2000). In this study, the velocity and amplitude of the wave tended to decline as it spread away from the origin, suggesting that diffusion of intracellular messengers, in concert with a regenerative process, may contribute to the overall wave propagation. The propagation of the wave front of the aspartate-induced wave could be well fitted to a diffusion equation (distance = $(6Dt)^{1/2}$, where D is the diffusion coefficient), which gave an average diffusion coefficient of 378 ± 35 μm^2/sec ($n = 15$). This is in good agreement with the known diffusion coefficient of IP_3 (283 μm^2/sec) measured in *Xenopus* oocytes (Allbritton et al., 1992). To our knowledge, the diffusion coefficient of cADPR has not been reported. The speed of wave propagation was not affected when either the IP_3- or the cADPR-mediated release was isolated (Fig. 4*C*), suggesting that their diffusion rates may be in the similar range.

Intracellular signaling cascade

Experiments with GDPβS and GTPγS confirmed the involvement of G-proteins. It is well established that the G_q family of G-proteins couples various neurotransmitter receptors, including group I mGluRs expressed in dopamine neurons, to the phospholipase C-IP_3 cascade (Hepler and Gilman, 1992). The synthesis of cADPR is catalyzed by the enzyme ADP-ribosyl cyclase; however, it is not known what subtype of G-proteins activates this enzyme. Recent evidence suggests that cell surface receptors can stimulate ADP-ribosyl cyclase activity in various mammalian cells (Cancela, 2001; Lee, 2001). In particular, the involvement of G-proteins was suggested in the cases of muscarinic receptor- and β-adrenergic receptor-mediated stimulation of ADP-ribosyl cyclase in NG108–15 cells and cardiac myocytes, respectively (Higashida et al., 1997, 1999).

In neurons, IP_3 acting on IP_3 receptors is thought to be solely responsible for the Ca^{2+} mobilization after activation of mGluRs and other G-protein-coupled receptors (Finch and Augustine, 1998; Takechi et al., 1998; Nakamura et al., 1999, 2000; Power and Sah, 2002). Previous studies in cultured cerebellar granule neurons have proposed that ryanodine receptor-mediated Ca^{2+} mobilization acts to amplify the IP_3-induced Ca^{2+} signal via Ca^{2+}-induced Ca^{2+} release (Irving et al.,

1992; Simpson et al., 1995, 1996). This proposal was based mainly on the inhibitory effect of ryanodine on mGluR- and muscarinic acetylcholine receptor-mediated Ca^{2+} signals, which were known to involve IP_3-dependent Ca^{2+} mobilization. However, ryanodine, which locks ryanodine receptor channels in an open state, may have caused depletion of IP_3-sensitive stores if those stores coexpressed ryanodine receptors (Khodakhah and Armstrong, 1997; Morikawa et al., 2000).

mGluR-mediated hyperpolarization and firing pattern

Dopamine neurons display a spectrum of activity ranging from pacemaker-like firing to burst firing *in vivo*. The burst firing is often followed by a pause of activity (Overton and Clark, 1997; Kitai et al., 1999). In contrast, they fire in a uniform pacemaker mode in a slice preparation, which is assumed to result from the loss of active synaptic inputs. In the present study, stimulation of glutamatergic fibers, as well as focal application of aspartate, reproduced the burst–pause pattern observed *in vivo*. A large body of evidence has implicated the glutamatergic inputs as the trigger for the burst, mainly by activating NMDA receptors (Overton and Clark, 1997; Kitai et al., 1999). In line with this, the burst was blocked by an NMDA receptor antagonist. In contrast, very few studies have addressed the mechanism of the pause, which has been explained mainly as a rebound phenomenon of the burst itself (Shepard and Bunney, 1991; Johnson et al., 1992). Our data show that the mGluR-induced hyperpolarization can mediate the pause independent of the burst.

Studies in monkeys have shown that burst firing of dopamine neurons is elicited by explicit reward-predicting stimuli. However, the pause of activity after the burst is promoted by presentation of stimuli that resemble, but are different from, these reward-predicting stimuli or some novel stimuli that are not necessarily related to rewards (Schultz, 1998). These data suggest that the pause by itself is caused by highly processed afferent inputs, rather than a rebound phenomenon of the burst. The present study proposes that a redundant mechanism activated by glutamatergic inputs ensures precise control of the firing pattern of dopamine neurons.

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